GENETIC ENGINEERING OF COTTON PLANTS AND LINES

Field of the Invention

The present invention relates to the general
technology of plant genetic engineering, and is related,
5   in particular, to a strategy for the transformation and
regeneration of cotton (Gossypium hirsutum L.) plants
using the Agrobacterium method of plant genetic
transformation so as to create novel genetically
transformed cotton plants and lines.

Background of the Invention

It is a general objective by many scientists working
in the field of plant biotechnology to successfully
genetically engineer plants of major crop varieties.
While plant genetic engineering has been successfully
15 demonstrated in several model plant species, often the
model plant species, such as tobacco, carrot and petunia,
are not the most economically important plant species for
agricultural purposes. Accordingly, much effort has been
directed toward the genetic engineering of the more
20 agriculturally important plant species. By the term
"genetic engineering" as used herein it is meant to
describe the introduction of foreign, often chimeric,
genes into one or more plant cells which can be
regenerated into whole, sexually competent, viable plants

which can be self-pollinated or cross-pollinated with other plants of the same species so that the foreign gene, carried in the germ line, can be inserted into or bred into agriculturally useful plant varieties.

5   The art of plant tissue culture has been an area of active research for many years but over the past five to ten years an intensified scientific effort has been made to develop regenerable plant tissue culture procedures for the important agricultural crops such as maize, wheat,
10 rice, soybeans, and cotton.

Early publications on tissue culture of cotton dealt mainly with establishing the growing tissues from the plant under aseptic conditions in vitro. Davis, D.G. et al. In Vitro 9:395-398 (1974); Rani, A. and S.S.
15 Bhojwani, Plant Sci. Lett. 7:163-169 (1976); and Price, H.J. et al., Plant Sci. Lett. 10:115-119 (1977). The methods detailed in these publications, however, did not provide the necessary framework needed to regenerate cells back into whole plants.
20 In the late 1970's, the development of somatic embryos, i.e., embryos derived from nongametic or somatic tissues, from the wild cotton species G. klotzchianum was reported. Price, H.J. and R.H. Smith, Planta 145:305-307 (1979). Unfortunately, there were two major problems
25 those using the procedure of this report were unable to overcome. First, even after several more years of research, these investigators could not induce the somatic embryos to germinate, i.e., convert, to give rise to whole plants, Finer, J.J. and R.H. Smith, Plant Cell Rep.
30 3:41-43, 1984. Secondly, the same technique could not be successfully replicated using cultivated cotton as a plant tissue source.

Davidonis and Hamilton were the first to report successful regeneration of whole plants from somatic
35 embryos of cotton. Davidonis, G.H. and R.H. Hamilton, Plant Sci. Lett. 32:89-93 (1984). These experimentors used

immature cotyledon tissues of the cultivar Coker 310. The
basic medium used consisted of Linsmaier and Skoog (LS)
salts, vitamins, and the phytohormones NAA and kinetin.
Linsmaier, E.M. and F. Skoog, Physiol. Plant. 18:100-127
5   (1965). The tissues used in this report had been used in
culture for several years without reports of similar
results, and the exact procedures necessary to replicate
this process are still not widely known.

     During this same year, the regeneration of plants from
10   several different California cotton cultivars was
reported, with a protocol sufficiently developed that it
could be replicated by the authors in reasonable time
periods. Rangan, T.S. et al., In Vitro 20:256 (1984). In
their procedure, several tissues, like cotyledons,
15   immature embryos, and hypocotyl tissue, were cultured on
Murashige and Skoog (MS) medium (Murashige, T. and F.
Skoog, Physiol. Plant. 15:473-497, 1962) plus the
phytohormones NAA and kinetin. After three-to-four months
of culture, these tissues gave rise to embryogenic callus
20   and somatic embryos. The embryos were then transferred to
a low salt medium, e.g., Beasley and Ting's (BT) medium
(Beasley, C.A. and I.P. Ting, Amer. J. Bot. 60:130-139,
1973) plus casein hydrolysate, which permitted some of
them to germinate and grow into whole plants. About 200
25   plants were recovered using the Acala SJ-5 cultivar. Some
sterility in the plants was observed and only 2% of the
plants showed somaclonal variation.

     Somatic embryogenesis was observed using the Coker
line 312 and a Texas race stock called T25. Robacker,
30   D.C. and T.W. Zimmerman, In the Ann. Mtg. of the American
Society of Agronomy, November 25-30, Las Vegas, NV. P. 85
(1984). The basal medium consisted of MS salts, the
vitamins inositol and thiamine, sucrose, and the
phytohormones NAA, 2,4 dichlorophenoxyacetic acid (2,4-D)
35   and kinetin. Hypocotyls were used as the original tissue
source. Even though embryos were recovered and cultured

onto BT medium, no plants were recovered.

Other investigators have also reported somatic embryogenesis and plant regeneration. (Trolinder, N.L. and J.R. Goodin, In the Proc. of the Beltwide Cotton

5 Production Research Conferences, January 6-11, 1985, New Orleans, LA. P. 46; and Mitten, D.H. In the Proc. of the Beltwide Cotton Production Research Conferences, January 6-11, 1985, New Orleans, LA. P. 57-58). These procedures were not published in detail, but based on the

10 presentations, enough data was collected such that certain themes began to emerge. One investigator achieved somatic embryogenesis using the Coker 310 cultivar, immature embryos, and hypocotyl tissues on MS medium plus the phytohormones NAA and 2iP (or kinetin). While clear

15 evidence was shown for somatic embryogenesis, the recovery of whole plants from these cultures was less clear. Other experimenters provided a clear and concise protocol. Repeatability was shown as was the recovery of intact plants. While they were able to obtain somatic embryos

20 from several different lines, the best cultivars belonged to the Coker pedigreed lines 5110 and 312 and one Texas race stock, T25. Other cultivars were unable to complete the regeneration process, i.e. convert to whole plants, or were unable to form mature somatic embryos. Basically,

25 their protocol used MS medium, B5 vitamins, and the phytohormones 2,4-D and kinetin.

These investigators have been focusing on plant regeneration of somatic non-transformed cotton tissues, but strategies directed toward the genetic engineering

30 plant lines typically generally involve two complementary processes. The first process involves the genetic transformation of one or more plant cells of a specifically characterized type. By transformation it is meant that a foreign gene, typically a chimeric gene

35 construct, is introduced into the genome of the individual plant cells, typically through the aid of a vector which

has the ability to transfer the gene of interest into the
genome of the plant cells in culture. The second process
then involves the regeneration of the transformed plant
cells into whole sexually competent plants. Neither the
5   transformation nor regeneration process need be 100%
successful, but must have a reasonable degree of
reliability and reproducibility so that a reasonable
percentage of the cells can be transformed and regenerated
into whole plants.
10   The two processes, transformation and regeneration,
must be complementary. It is possible to transform
certain tissues or cell types which cannot be regenerated,
and it is also possible to regenerate plant tissues of a
number of different tissue and cell types which have not
15 yet been successfully transformed, as demonstrated by the
investigators discussed above. The complementarity of the
two processes must be such that the tissues which are
successfully genetically transformed by the transformation
process must be of a type and character, and must be in
20 sufficient health, competency and vitality, so that they
can be successfully regenerated into whole plants.
Successful transformation and regeneration techniques
have been demonstrated in the prior art for other plant
species. For example, in Barton et al., "Regeneration of
25 Intact Tobacco Plants Containing Full-Length Copies of
Genetically Engineered T-DNA, and Transmission of DNA to
R 1 Progeny", Cell 32:1033 (April 1983), the
transformation and regeneration of tobacco plants was
reported. Similar results have been achieved in some
30 other plant species, though not cotton.
The most common methodology used for the
transformation of cells of dicot plant species involves
the use of the plant pathogen Agrobacterium tumefaciens.
A. tumefaciens harbors a plasmid, referred to as the
35 tumor-inducing or Ti plasmid, which has the natural
ability to transfer a segment of itself, referred to as

the T-DNA (transfer-DNA), into the genome of infected
plant cells. Wild-type A. tumefaciens use this ability to
genetically transform infected cells of plants so that the
plant cells become tumorous, and also synthesize one of a
5   series of compounds, known as opines, which can be
metabolized by the infecting A. tumefaciens. It has been
found by several investigators that by removing the bulk
of the T-DNA from a Ti plasmid harbored by A. tumefaciens,
and by replacing that T-DNA with a foreign gene
10 construction, that the Agrobacterium can transform
infected plant cells with the foreign gene in such a
fashion that the resultant cells are not tumorous, as
plant cells infected with wild-type normally A.
tumefaciens are. The foreign gene construction is then
15 included in the cells of a whole plant regenerated from
the transformed cells and is then inherited in a simple
Mendelian manner. The construction can thus be treated as
any inheritable trait for crop breeding purposes.

Although the regeneration of whole plants from somatic
20 embryos of cotton has been previously reported, it has not
been heretofore thought to have been possible to
genetically form whole cotton plants utilizing the
transformation/regeneration techniques.

It is therefore an object of the present invention to
25 genetically engineer whole intact cotton plants and lines.

It is further an object of the present invention to
genetically engineer the production of whole cotton plants
utilizing transformation/regeneration techniques.

It is still further an object of the present invention
30 to genetically engineer the production of whole cotton
plants, using the Agrobacterium method of gene
transformation followed by a reproducible regeneration
technique.

These objects and others are fulfilled by the present
35 invention as described below.

Summary of the Invention

The present invention involves the transformation of cotton cells in tissue culture and the regeneration of those cells into whole sexually mature cotton plants. It has been found that tissues derived from hypocotyl pieces of immature cotton plants can be inoculated with a culture of A. tumefaciens without adversely affecting the ability to elicit regenerable somatic embryo generation. Through the use of selectable markers, it is possible to screen, in the tissue culture stage, for transformed clonal growth of regenerable tissues to obtain whole plant development.

In brief, the process involves the steps of inoculating (or infecting) the hypocotyl pieces of immature cotton plants with an A. tumifaciens strain; tissue culture plating of the infected tissues; removing the bacteria from the infected tissues and simultaneously selecting tissues resistant to a selectable marker; amplifying the resistant tissues; and regenerating into whole plants.

The present invention is thus useful for the general genetic engineering of cotton plants and lines through the insertion into the germ line of such plants selected foreign genes.

Description of the Figures

Figure 1 is a schematic restriction enzyme mapping illustration of plant carrier plasmid pCMC92.

Fig. 2 is a restriction enzyme mapping illustration of the plant transformation plasmid pCMC1204.

Detailed Description of the Invention

The method of genetically engineering cotton plants and lines according to the present invention can, as stated above, be broadly thought of as involving two related processes. The first process is the genetic transformation of cotton cells or tissues in tissue

culture. The second broad process is the regeneration of
intact, sexually mature cotton plants from genetically
transformed cells. The two processes are interdependent
in that the tissues which are transformed in the
5   regeneration process must be of a kind and of a competence
so that they can be regenerated through the appropriate
regeneration process. The present process envisions the
genetic transformation of tissues in culture derived from
hypocotyl explants excised from cotton plants four to six
10 days after germination. The transformed tissues derived
from the hypocotyl pieces can be induced to form embryonic
structures, referred to here as somatic embryos, which can
be regenerated into whole, relatively normal, and sexually
competent cotton plants.

15   The transformation technique of the present invention
is one which makes use of the Ti plasmid of A.
tumefaciens. In using an A. tumefaciens culture as a
transformation vehicle, it is most advantageous to use a
non-oncogenic strain of the Agrobacterium as the vector
20 carrier so that normal non-oncogenic differentiation of
the transformed tissues is possible. It is also
preferred, as a matter of convenience in preparing the
actual genetic vector for transfer into the plant cells,
that the Agrobacterium harbor a binary Ti plasmid system.
25 In such a binary system, the Agrobacterium carries a first
Ti plasmid having a virulence region therein and a second,
chimeric plasmid, which contains therein the border
regions of the T-DNA region of a wild-type Ti plasmid
surrounding a chimeric gene construction including a
30 foreign gene of interest. Binary Ti plasmid systems have
been shown effective to transform plant cells. De
Framond, Biotechnology, 1:262-269 (1983); Hoekema et al.,
Nature, 303:179 (1983). Such a binary system is preferred
because the smaller plasmid, containing the T-DNA borders,
35 can be constructed and manipulated most easily in an
alternative host, such as E. coli, and then transferred

into Agrobacterium. To be effective once introduced into cotton cells, the chimeric construction including foreign gene of interest must, in addition to a heterologous gene coding for a product of interest, contain a promoter which

5 is effective in cotton cells to cause transcription of the gene of interest and a polyadenylation sequence or transcription control sequence also recognized in cotton cells. Promoters known to be effective in cotton cells include the nopaline synthase promoter, isolated from the

10 T-DNA of Agrobacterium, and the cauliflower mozaic virus 35s promoter. It is also preferred that the binary plasmid harboring the foreign gene of interest also contained therein one or more selectable marker genes so that the transformed cells can be selected from

15 non-transformed cells in culture. Preferred marker genes include antibiotic resistance genes so that the appropriate antibiotic can be used to segregate and select for transformed cells from among cells which are not transformed.

20 The heterologous gene selected for insertion into the cotton tissues can be any foreign gene selected to achieve a useful result if expressed in cotton plants. For example, expression of the Bacillus thuringiensis crystal protein toxin in the cells of cotton plants would make

25 those cells toxic if ingested by Lepidoptera insects, thus inbuing the plants with a useful pest resistance. Expression of a viral coat protein in the cotton plant cells would make the plant resistant to infection by that virus. Transcription of negative strand RNA's in cotton

30 plant cells can be useful for inhibition of undesirable endogenous genes or for disease resistance traits. As is understood by those skilled in the art, the demonstration here of the expression of a foreign protein in a cotton plant can readily be repeated with any other foreign gene

35 of interest.

To commence a transformation process in accordance

with the present invention, it is first necessary to construct the foreign genes of interest and properly introduce them into a transformation competent, but non-oncogenic, strain of <u>Agrobacterium</u> <u>tumefaciens</u>. The

5 details of the construction of the vectors containing such foreign genes of interest are known to those skilled in the art of plant genetic engineering and do not differ in kind from those practices which have previously been demonstrated to be effective in tobacco, petunia, and

10 other model plant species. The foreign gene should obviously be selected to accomplish some desirable effect in cotton plant cells. This effect may be growth promotion, disease resistance, a change in plant morphology or plant product (i.e. lint) quality, or any

15 other change which can be accomplished by genetic manipulation. The chimeric gene construction can code for the expression of one or more exogenous proteins, or can cause the transcription of negative strand RNAs to control or inhibit either a disease process or an undesirable

20 endogenous plant function.

To initiate the transformation and regeneration process for cotton plants, it is necessary to first surface sterilize cotton seeds to prevent inadvertent contamination of the resulting culture. The seeds are

25 then allowed to germinate on an appropriate germinating medium containing a fungicide.

Four to six days after germination the hypocotyl portion of the immature plant is removed and sectioned into small segments averaging approximately 0.5

30 centimeters apiece. The hypocotyl explants are allowed to stabilize and remain viable in a liquid or agar plant tissue culture medium.

Once the hypocotyl segments have stabilized, they can promptly be inoculated with a suspension culture of

35 transformation competent non-oncogenic <u>Agrobacterium</u>. The inoculation process is allowed to proceed for three to

five days at room temperatures, i.e., 24° C.

At the end of the inoculation time period, it is necessary first to rinse off the excess Agrobacterium. Then the remaining treated tissues can be transferred to a

5 second agar medium, which also contains one or more antibiotics toxic to Agrobacterium, but not to hypocotyl tissues, at a concentration sufficient to kill any Agrobacterium remaining in the culture. Suitable antibiotics for use in such a medium include carbenicillin

10 and cefotaxime. The tissues are then given a period of from one to ten days to recover from the transformation process and are then continued in culture.

The tissues are now cultivated on a tissue culture medium which, in addition to its normal components,

15 contains a selection agent, the selection agent being one toxic to non-transformed cotton cells but not to transformed cotton cells which have incorporated genetic resistance to the selection agent and are expressing that resistance. A suitable tissue culture medium is the MS

20 medium to which is added the phytohormones 2,4 dichlorophenoxy-acetic acid (2-4,D), 6-furfurylaminopurine and a gelling agent. Suitable selection agents include both antibiotics and herbicides. Suitable antibiotic traits which may serve as dominant selectable markers

25 include the aminoglycoside phosphotransferase-3'-II (APH-(3')-II) gene, also referred to as the neomycin phosphotransferase II gene (NPTII), which code for resistance to the antibiotic kanamycin, and the APH-(3')-IV gene which codes for resistance to Hygromycin

30 B. Kanamycin, G418 and Hygromycin B are aminoglycosides that will stop the growth of non-transformed cotton cells, but these antibiotics are phosphorylated by the appropriate enzyme if it is expressed in the transformed cells. Another suitable antibiotic selection agent is

35 Chloramphenicol which can be acetylated and rendered non-toxic by the enzyme produced by the CAT

(Chloramphenicol acetyl transferase) gene. Antibiotic
dosed media allows only transformed cells to continue to
grow and thrive. Thus the transformed cells, or calli,
are allowed to grow on the selective medium. The
5 surviving transformed tissues are transferred to a
secondary medium to induce somatic embryogenesis. The
surviving transformed tissue will thus continue to form
into somatic embryos, which can then be regenerated
through the regeneration technique of the present
10 invention or through any other alternative plant
regeneration protocols which use cotton somatic embryos as
their starting point.

The selection process should continue for an extended
time, i.e. 3-4 months, because of the slow growth of even
15 transformed tissues on the antibiotic medium. Subcultures
are made every 4-6 weeks to replenish nutrients and
antibiotics. As the transformed cells are selected and
amplified, individually derived cell lines are
identifiable and can be removed and separately amplified.

20 The regeneration technique in accordance with the
present invention begins with the tissues resulting from
the transformation process. These tissues are putatively
transformed calli which can generate somatic embryos when
cultivated on appropriate embryo induction media. One
25 technique for regenerating these somatic embryos to whole
plants is disclosed here, but it is to be understood that
other techniques are also possible, once transformed
embryogenic tissues are produced.

The regeneration technique used by the applicants here
30 thus begins with the tissues resulting from the
transformation process. The cotton tissue calli,
generated from the hypocotyl segments of the cotton
plants, and putatively transformed, are placed onto
somatic embryo induction media directly. At this point,
35 the antibiotic selection agent should be removed from the
culture medium, but otherwise the medium may remain

constant. These calli, cultured on the somatic embryo
induction medium, will form small embryoidal structures,
which have been termed somatic embryos. It may take as
long as two to three months for the somatic embryos to
5   emerge and mature. Approximately 5 to as many as 20
somatic embryos will emerge from a single callus in an
agar formulation of a somatic embryo induction medium.
Many of the somatic embryos thus produced will be
regenerable into whole plants in accordance with the
10 technique described here.

When the developing somatic embryos are large enough,
i.e. to a size of 4 mm or more in length, and if they
appeared to have good embryonic development, i.e. usually
having a cotyledon and a radicle, they may be transferred
15 to large test tubes and hosted on fine vermiculite. The
vermiculite is saturated with Stewart and Hsu (SH) medium
(Planta 137:113 (1977)) plus the phytohormones indole
acetic acid, 6-furfurylaminpurine and gibberellic acid.
Small plantlets, having two to three leaves, eventually
20 develop.

Once plantlet growth is established, i.e., the 2-3
leaf stage, the plants can now move into plant pots with
vermiculite soil. They may be watered and fertilized as
needed. They may also need to be hardened off, before
25 greenhouse exposure. The plantlets may be repotted when
they have 4-6 leaves after which they will continue to
grow until mature.

The following examples will serve to illustrate the
process of the invention without limiting the same.

30   EXAMPLE 1
        Transformation of Callus
This example was designed to illustrate the
transformation of cotton tissues in culture using virulent
and avirulent strains of A. tumefaciens which carried
35 plasmids that encoded for the marker enzymes neomycin

phosphotransferase II (NPTII) (also known as APH-3'-II)
and/or chloramphenicol acetyl transferase (CAT). The
callus tissues were amplified over a period of several
months on a selection medium. Tissue samples were removed
5   and assayed for expression of the enzymes and the
remaining tissues were used for DNA analysis. The details
of the example are as follows:

Seeds of cultivated cotton (Gossypium hirsutum L.) of
Coker lines 310, 312, 5110 Deltapine 61, and Stoneville
10 213 were surface sterilized with 3% sodium hypochlorite
for twenty minutes. The seeds were then rinsed three
times with sterile distilled water plus cefotaxime (500
mg/l). The seeds were allowed to germinate in SH medium
containing the fungicide benomyl (50 mg/l). Four to six
15 days after germination, hypocotyl explants were removed,
cut into 0.5 cm pieces, and placed on a support medium
containing agar (0.8%) and water.

The hypocotyl pieces were then inoculated with diluted
(1:10) overnight culture of A. tumefaciens. The
20 suspension culture of A. tumefaciens contained
approximately 10^8 bacteria per milliliter.

The particular A. tumefaciens strain used harbored a
binary Ti plasmid system containing one Ti plasmid
carrying the so-called virulence region and a second Ti
25 plasmid containing T-DNA border regions from a virulent Ti
plasmid (pCMC91, pCMC92 or pCMC1204) and also containing
an antibiotic resistance gene (NPT II). One plasmid
(pCMC1204) also carried a foreign gene (CAT) between the
T-DNA borders. The infection of the A. tumefaciens on the
30 immature embryonic tissues was allowed to proceed for
three to five days at room temperature.

After incubation at room temperature and lighting, the
tissues were transferred to Murashige and Skoog salts with
B5 vitamins (Gamborg et al., Exp. Cell Res. 50:151-158
35 (1968)) plus antibiotics, the phytohormones 2,4-D (0.1
mg/l) and 6-furfurylaminopurine (0.1 mg/l), and the

gelling agent Gelrite (1.6 g/l) (Gelrite is a registered
trademark of Kelco Company, San Diego, California) plus
magnesium chloride (750 mg/l). The antibiotics used to
kill the bacteria were cefotaxime (50-100 mg/l) and
5 carbenicillin (400-500 mg/l). Kanamycin sulfate (5-50
mg/l) was included in the medium and used as a selective
agent. Subcultures were made every four to six weeks to
replenish depleted nutrients and antibiotics.

After three to four months, individually derived cell
10 lines were labelled and maintained on a selection medium
for tissue amplification. Tissues were incubated at 30° C
with a 16 hour photoperiod (50-100 umol/m^2/s).

NPTII assays were carried out using crude cell
extracts prepared from calli or plant tissues preincubated
15 with a buffer solution (30 mM NaCl, 15 mM NH$_2$Cl, 3 mM
MgCl, 5.5 mM Na EDTA, 2.3 mM Tris, 0.2 mM dithiothreitol
(DTT), 105 mM sucrose at a pH of 7). Following three
freeze-thaw cycles in liquid nitrogen, tissues were ground
in buffer containing 37 mM Na EDTA, 126 mM NaCl, 83 mM
20 NH$_2$Cl, 7.7 mM Tris, and 15 mM DTT. To protect against
protease degradation of NPTII, 2.5 mM phenylmethylsulfonyl
fluoride and 0.6 mg/ml leupeptin, 1.05 mg/ml soybean
trypsin inhibitor and 5.0 mg/ml bovine serum albumin were
added to the buffer. Forty microliters of extraction
25 buffer was used per 100 mg (fresh weight) of callus or
plant tissue. Supernatents were collected following
centrifugation. Electrophoretic analysis of NPTII
activity was carried out according to Paszknowski, J. et
al., EMBO. J. 3:2717-2722 (1984); and Reiss, B. et al.,
30 1984, Gene, 30:211-218 (1984).

Plant tissues were extracted according to the
procedure of Herrera-Estrella et al., Nature, 303:209-213
(1983). The extracted plant tissues were CAT assayed as
described by Gorman, C.M. et al., Molec. Cell. Biol.
35 2:1044-1051 (1982).

Table 1 lists the various vectors that were tested,

the number of transformed lines obtained and the result of their NPTII test. Table 2 describes data collected for samples of two of the vectors.

Table 1

Vector constructions that have been tested in cotton

Vector	No. of Trans-formed Lines Recovered	No. of Lines Tested for NPTII	No. of Lines With (+) Assays	%
A6	4	4	0	0
A6(pCMC91)	21	21	16	76
A6(pCMC92)	16	16	16	100
LBA4404(pCMC91)	17	17	15	88
LBA4404(pCMC92)	4	4	4	100
LBA4404(pCMC1204)	40	6	5	83

The A6 construction does not carry the Kanamycin resistance gene and, therefore, will not produce the NPTII protein. A6 is a virulent Agrobacterium strain. LBA4404 is a non-virulent strain. Plasmids pCMC91 and pCMC92 carry the NPT II gene alone between the T-DNA border segments. Plasmid pCMC1204 carries the NPT II and CAT genes in tandem.

Table 2

NPTII activity in transformed cotton callus

Vector/Sample	cpm/20 ul crude extract	ng enzyme/20 ul *
Controls:		
Stoneville 231	102	L 0.1
Coker 310	0	L 0.1
Deltapine 61	25	L 0.1
Vector LBA4404(92)		
1	26,320	3.06
2	77,016	8.97
3	31,190	3.63
4	53,413	6.20

Nanogram values were calculated from counts obtained from a known amount of NPTII enzyme run on the same gel. "L" indicates less than.

The results indicate the expression of at least one
enzyme (NPTII) and/or in most cases the expression of both
enzymes (NPTII and CAT) when they were incorporated
together on the same plasmid in tissues of cotton callus

5   in culture.

EXAMPLE 2

Transformation and Regeneration

In this example, cotton tissues were transformed using
an avirulent strain of A. tumefaciens (LBA4404) which

10   carried a plasmid (pCMC1204) that encoded for the marker
enzyme NPTII and also for CAT. Over the next several
months callus tissues were amplified on selection medium.
Tissue samples were removed and assayed for expression of
the enzymes. The remaining callus tissues were used to

15   regenerate plants. Details of the example follow.

The plasmid pCMC1204 was constructed from components
of a bipartite vector system similar to those previously
described in the art. de Framond et al., Bio/technology,
1:262-269 (1983), Bevan, Nuc. Acids Res, 12:8711-8721

20   (1984). An expression cassette for the CAT gene was
prepared by ligating the Hind III/Sau 3Al CAT coding
fragment from pSV2CAT (Gorman et al. Mol. Cell. Biol.
2:1044-1051 (1982)) into the Hind III and Bam HI sites of
pCMC66. The plasmid pCMC66 is an expression plasmid which

25   employs the promoter and polyadenylation regions from the
nopaline synthase nos gene of pTiT37 of Agrobacterium.
The resulting CAT expression plasmid, designated pCMC1201,
was linearized with Sal I which cuts the plasmid once
immediately downstream of the polyadenylation region, and

30   was inserted into the unique Sal I site on the carrier
plasmid pCMC92. The carrier plasmid pCMC92 is a wide-host
range replicon carrying the T-DNA border regions from
Agrobacterium and a chimeric NPTII gene. The chimeric
NPTII gene is formed from the nopaline synthase promoter

35   and polydenylation sequence from pTiT37, ie. the same
control sequences as in pCMC66, and the NPTII coding

region from Ti5. The product of the ligation was
designated pCMC1204. Plasmid pCMC1204 was mobilized from
E.Coli into A. tumefaciens strain LBA4404 in a triparental
mating with a helper strain of E.Coli carrying pRK2013.

5   Transconjugants were selected on plates containing
streptomycin, sulfadiozone and carbenicillin at 100
microgram/ml. Single colonies were picked for
amplification for inoculation of plant tissues.

     The construction and use of both plasmids pCMC66 and

10   pCMC92 is described in more detail in published patent
application WO 85/04899 by Barton and Gelfand. The
plasmid pCMC92 is on deposit with the ATCC, accession
number 53093 and not only contains the chimeric NPTII gene
and T-DNA borders, but contains the nopaline synthase

15   promoter and polyadenylation sequences which can be
removed from that plasmid and used with any desired
heterologous coding sequence.

     Hypocotyl pieces were inoculated with diluted (1:10)
overnight cultures of A. tumefaciens. After three days

20   incubation at room temperature (23° - 27°C) and lighting,
the tissues were transferred to MS salts with B5 vitamins
plus antibiotics, the phytohormones 2,4-D (0.1 mg/l) and 6
furfurylaminopurine (0.1 mg/l) and the gelling agent
Gelrite (1.6 g/l), plus magnesium chloride (750 mg/l).

25   The antibiotics used to kill the bacteria were cefotaxime
(50-100 mg/l) and carbenicillin (400-500 mg/l). Kanamycin
sulfate (5-50 mg/l) was included in the medium and used as
a selective agent. Subcultures were made every four to
six weeks to replenish depleted nutrients and

30   antibiotics. After three to four months, individually
derived cell lines were labeled and maintained on the
selection medium for tissue amplification. The tissues
were incubated at 30°C for a 16 hour photoperiod (50-100
umol/m^2/s).

35   After amplification, the antibiotics were discontinued
and the transformed tissues were maintained on the same

medium. After an additional 2 to 3 months, embryogenic calli emerged to give rise to somatic embryos. When the embryos were large enough, i.e., 4 mm or more in length, and appeared to have good embryo development, cotyledon

5   and radicle present, they were transferred to large test tubes containing "fine" vermiculite. The vermiculite was saturated with SH medium plus the phytohormones indole acetic acid, 6 furfurylaminopurine and gibberellic acid, all at 0.1 mg/l. The embryos were incubated at a 30°C, 16

10   hour photoperiod. Upon germination, i.e., 2-3 leaf stage, plantlets were transferred to pots filled with vermiculite and watered and fertilized as needed. The plantlets were enclosed in a beaker for about one week to help harden-off the leaves. Once potted-plantlet growth was established

15   (4-6 leaf stage), they were moved to the greenhouse. Adapted plants were repotted in a commercial soil mixture, Metro Mix 360 (Metro Mix is a registered trademark of W. R. Grace and Co.), and maintained until mature.

The NPTII assays were performed according to the

20   process outline in Example 1. The assays indicated NPTII activity in the leaf tissue of several of the regenerated cotton plants. Some plants grown from callus testing positive for NPTII activity tested negative, however.

To confirm the presence of the plasmid in the

25   transformed plants, a Southern blot analysis was performed. The Southern blot analysis was performed with the restriction enzymes Ava I and Hind III, corresponding to the NPTII coding region. The plant DNA, extracted and digested, was hybridized with a radiolabeled negative

30   strand NPTII sequence. A hybridizing band indicated presence of the expected fragment in the plant tissues in at least one copy number.

The plasmid pCMC 1204 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville,

35   MD, USA, on December 3, 1986 under ATCC accession No. 67275 .

The above deposit was made pursuant to a contract between the ATCC and the Cetus Corporation, a partner in the assignee of the present invention. The contract with the ATCC provides for permanent availability of the progeny of these cell lines to the public on the issuance of the US patent describing and identifying the deposit or the publication or laying open to the public of any US or foreign patent application, whichever comes first, and for availability of the progeny of these cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules prusuant thereto (including 37 CFR Section 1.14 with particular reference to 886 O.G. 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable culture of the same cell line.

The present invention is not to be limited in scope by the microorganisms deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

Although the present invention has been described with preferred embodiments, it is to be understood that modifications and variations may be resorted to, without departing from the spirit and scope of this invention, as those skilled in the art will readily understand.